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L10: Entry 10 of 24   File: PGPB   Jan 24, 2002

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TITLE: Method for loading cells with an agent

PUBLICATION-DATE: January 24, 2002

INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/2; 435/810

CLAIMS:

1. A method for selectively releasing an agent from a red blood cell comprising the steps of: (a) presensitising said red blood cell; (b) loading said red blood cell with an agent; (c) electrosensitising said red blood cell; and (d) causing said agent to be released from said sensitised red blood cell by applying ultrasound to cause disruption of said sensitised red blood cell, wherein steps (b) and (c) are performed in any order.

2. A method according to claim 1 wherein said electrosensitisation procedures in step (a) and (c) are in vitro or ex-vivo procedures.

3. A method according to claim 1 or claim 2 wherein said electrosensitisation comprises the step of applying an electric field to the red blood cell.

4. A method according to claim 3 wherein said electric field applied to said red blood cells ranges from 0.1 kV/cm to 10 kV/cm under in vitro conditions.

5. A method according to claim 4 wherein said electric field is applied to said red blood cell for 1 .mu.s to 100 ms.

6. A method according to claim 1 wherein said ultrasound is selected from the group consisting of diagnostic ultrasound, therapeutic ultrasound and a combination of diagnostic and therapeutic ultrasound.

7. A method according to claim 6 wherein the applied ultrasound

energy source is at a power level that ranges from 0.05 W/cm.sup.2 to about 100 W/cm.sup.2.

8. A method for delivering an agent in a vertebrate, comprising the steps of: (a) presensitising a red blood cell; (b) loading said red blood cell with an agent; (c) electrosensitising said red blood cell; (d) introducing said red blood cell into said vertebrate; and (e) causing said agent to be released from said sensitised red blood cell by applying ultrasound to cause disruption of said sensitised red blood cell, wherein steps (b) and (c) are performed in any order.

9. A method according to claim 8, wherein said red blood cell is PEGylated prior to being introduced into said vertebrate.

10. A method according to claim 8, wherein said vertebrate is a mammal.

11. A method according to claim 1 or 8 wherein said sensitisation of said red blood cell in step (c) is performed after the loading of said agent in step (b).

12. A method according to claim 1 or 8 wherein said sensitisation of said red blood cell in step (c) is performed before the loading of said agent in step (b).

13. A method according to claim 1 or 8 wherein said loading is performed by osmotic shock.

14. A method according to claim 1 or 8 wherein said agent is selected from a group consisting of a protein, a polypeptide, a peptide, a nucleic acid, a virus, a virus-like particle, a nucleotide, a ribonucleotide, a deoxyribonucleotide, a modified deoxyribonucleotide, a heteroduplex, a nanoparticle, a synthetic analogue of a nucleotide, a synthetic analogue of a ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analogue, a modified amino acid, a modified amino acid analogue, a steroid, a proteoglycan, a lipid, an oligosaccharide, a glycoprotein and a carbohydrate.

15. A method for preparing a red blood cell composition comprising: (a) presensitising said red blood cell; (b) loading said red blood cell with an agent; and (c) electrosensitising said red blood cell, wherein steps (b) and (c) are performed in any order.

16. A red blood cell composition obtainable by a method comprising (a) presensitising said red blood cell; (b) loading said red blood cell with an agent; and (c) electrosensitising said red blood cell, wherein steps (b) and (c) are performed in any order.

17. A kit comprising the red blood cell composition of claim 16, packaging materials therefor and instructions for use.

18. A kit comprising a red blood cell, an agent, packaging materials therefor and instructions for use in a method comprising the steps of: (a) presensitising said red blood cell; (b) loading said red blood cell with said agent; (c) electrosensitising said

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L10: Entry 11 of 24   File: PGPB   Dec 20, 2001

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TITLE: Loading method

PUBLICATION-DATE: December 20, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
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Craig, Roger	Smallwood		GB	
Fadlon, Emma Jane	Portstewart		GB	

US-CL-CURRENT: 435/446; 435/173.1

CLAIMS:

We claim:

1. A method of producing a red blood cell comprising an agent comprising: (a) providing said red blood cell; (b) pre-sensitising said red blood cell; and, (c) loading said red blood cell with said agent.

2. A method according to claim 1 said step (c) comprising leading a first and said red blood cell with a second agent.

3. A method according to claim 1, further comprising the step of electrosensitising the cell.

4. A method for selectively releasing an agent from a red blood   C 1,4,8
cell comprising the steps of: (a) pre-sensitising a red blood cell; (b) loading said red blood cell with an agent; (c) electrosensitising said red bood cell; and (d) effectuating substantial release of said agent from said sensitised red blood cell by applying ultrasound. at a frequency and energy sufficient to cause disruption of unsensitised red blood cells.

5. A method for delivering an agent in a vertebrate comprising, (a) pre-sensitising a red blood cell; (b) loading said red blood cell   1,4,8
with an agent; (c) electrosensitising said red blood cell; (d) introducing said red blood cell into a vertebrate; and (d) releasing said agent from said sensitised cell by ultrasound.

6. A method according to claim 5, wherein said red blood cell of

step (w) is immunocompatibile with said vertebrate.

7. A method according to claim 5, in which the red blood cell is   16
PEGylated prior to being introduced into the vertebrate.

8. A method according to claim 5 in which the vertebrate is a
mammal.

9. A method according to claim 1 or claim 5 wherein one or both of   9
said pre-sensitising or electrosensitising steps is performed in
vitro or ex-vivo.

10. A method according to claim 1 or claim 5, wherein said
pre-sensitising step comprises applying an electric field to said
red blood cell.

11. A method according to claims 1 or claim 5, wherein said
pre-sensitising step further comprises applying ultrasound to the
red blood cell.

12. A method according to claim 1 or claim 5, wherein said loading   21?
step comprises hypotonic dialysis.

13. A method according to claim 3, wherein said electrosensitizing
step comprises applying an electric field to said red blood cell.

14. A method according to claim 13, wherein said electric field   3, 11
applied to said red blood cell ranges from 0.1 kV/cm to 10 kV/cm.

15. A method according to claim 13, wherein said electric field is   2, 12
applied to said red blood cell 1 microsecond to 100 milliseconds.

16. A method according to claim 3, wherein said
electrosensitisation step is performed after said loading step.   6, 20

17. A method according to claim 3, wherein said
electrosensitisation step is performed before said loading step.   5, 19

18. A method according to claim 4, wherein said ultrasound is   13
selected from the group consisting of diagnostic ultrasound,
therapeutic ultrasound and a combination of diagnostic and
therapeutic ultrasound.

19. A method according to claim 4 wherein the applied ultrasound   14
energy source is at a power level from about 0.05 W/cm.sup.2 to
about 100 W/cm.sup.2.

20. A red blood cell composition comprising a plurality of
pre-sensitized red blood cells.

21. The red blood cell composition according to claim 20, wherein
said red blood cell is pre-sensitized to permit loading of an
agent.

22. A red blood cell composition according to claim 20 comprising a
plurality of pre-sensitized electro sensitized red blood cells.

23. A red blood cell composition according to claim 20, wherein said red blood cells are immunocompatible in a vertebrate.

24. A red blood cell composition according to claim 20 wherein said agent is selected from a group consisting of: a protein, a polypeptide, a peptide, a nucleic acid, a peptide nucleic acid (PNA), a virus, a nucleotide, a ribonucleotide, a deoxyribonucleotide, a heteroduplex, a nanoparticle, an amino acid, a steroid, a proteoglycan, a lipid, a fatty acid, an oligosaccharide, a glycoprotein, and a carbohydrate.

25. A red blood cell composition according to claim 24 wherein said agent further comprises an imaging agent.

26. A red blood cell composition obtainable by a method comprising: (a) presensitising a red blood cell; (b) loading the cell with an agent; and (c) electrosensitising the cell.

27. A kit comprising a red blood cell composition according to claim 20, and packaging materials therefor.

28. A kit comprising a pre-sensitised red blood cell, an agent, and packaging materials therefor.

29. A kit according to claim 27 or 28, said kit further comprising a liquid selected from the group consisting of: a buffer, diluent, an excipient, a saline buffer, a physiological buffer, serum, and plasma.

30. A pharmaceutical composition comprising a red blood cell composition made by a process comprising: (a) providing a red blood cell; (b) pre-sensitizing said red blood cell; (c) loading said red blood cell with an agent; and (d) electrosensitizing said red blood cell.

31. The composition of claim 31 wherein said red blood cell composition further comprises a red blood cell is immunocompatible in a vertebrate.

32. The composition of claim 31 wherein said red blood cell comprises PEG.

33. A device for producing a red blood cell delivery composition, comprising: (a) one or more flow cells and electrosensitisation means; (b) one or more dialysis systems; in which the flow cell is linked to the dialysis system by connecting means capable of allowing transfer of red blood cells from the flow cell to the dialysis system.

34. A device as claimed in claim 33 wherein said device for sensitizing said red blood cell emits an electric field.

35. A device as claimed in claim 33 wherein said device for sensitizing said red blood cell emits ultrasound.

(FILE 'HOME' ENTERED AT 15:04:05 ON 11 SEP 2003)

FILE 'MEDLINE' ENTERED AT 15:04:11 ON 11 SEP 2003
L1   118 SEA PLU=ON (RED BLOOD CELL OR ERYTHROCYTE) AND (ULTRASON? OR
          ULTRASOUND? OR SONIF? OR SONIC?) AND (LYSE OR LYSIS OR
          DISRUPT? OR RUPTUR?)
L2   3 SEA PLU=ON L1 AND (SENSITIS? OR SENSITIZ? OR
ELECTROSENS?)
          D BIB AB 1-3

FILE 'CAPLUS, EMBASE, BIOSIS, BIOTECHDS' ENTERED AT 15:08:59 ON 11
SEP
   2003
L3   0 SEA PLU=ON CAPLUS EMBASE BIOSIS BIOTECHDS SCISEARCH

FILE 'CAPLUS, EMBASE, BIOSIS, BIOTECHDS, SCISEARCH' ENTERED AT
15:09:28
   ON 11 SEP 2003
L4 .   16 SEA PLU=ON L2
L5   12 DUP REM L4 (4 DUPLICATES REMOVED)
          D TI 1-12
          D BIB AB 1-5

FILE 'MEDLINE, CAPLUS, EMBASE, BIOSIS, BIOTECHDS, SCISEARCH'
ENTERED AT
   15:14:11 ON 11 SEP 2003
L6   509 SEA PLU=ON MCHALE A?/AU
L7   4337 SEA PLU=ON CRAIG R?/AU
L8   258 SEA PLU=ON HARO A?/AU
L9   5096 SEA PLU=ON L6 OR L7 OR L8
L10   70 SEA PLU=ON L9 AND (ERYTHROCYTE OR RED BLOOD CELL)
L11   27 SEA PLU=ON L10 AND (LYSE OR LYSIS OR DISRUPT? OR
RUPTUR?)
L12   10 DUP REM L11 (17 DUPLICATES REMOVED)
          D BIB 1-10
          D BIB AB 7-10
L13   5279 SEA PLU=ON (RED BLOOD CELL OR ERYTHROCYTE) AND
(SONIF? OR
          SONIC? OR ULTRASON? OR ULTRASOUND)
L14   521 SEA PLU=ON L13 AND L1
L15   276 DUP REM L14 (245 DUPLICATES REMOVED)
          D BIB 50 100 150
L16   13 SEA PLU=ON L15 AND L2
          D TI BIB 1-13

From: Schnizer, Richard
Sent: Thursday, September 11, 2003 3:36 PM
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Please sed me copies of:

TI   Electric field-enhanced activation of hematoporphyrin derivative: effects
     on a human tumour cell line.
AU   Ward T; Mooney D; Flynn G; McHale A P
SO   CANCER LETTERS, (1997 Feb 26) 113 (1-2) 145-51.

TI   Use of real-time confocal laser scanning microscopy to study immediate
     effects of photodynamic activation on photosensitized erythrocytes
AU   Rollan A; Ward T; Flynn G; McKerr G; McHale L; McHale A P

SO   CANCER LETTERS, (1996 Mar 29) 101 (2) 165-9.

TI   The effects of electric fields on photosensitized erythrocytes:
     possible enhancement of photodynamic activation.
AU   Ward T; Rollan A; Flynn G; McHale A P
SO   CANCER.LETTERS, (1996 Aug 23) 106 (1) 69-74.

TI   Encapsulation of the thrombolytic enzyme, brinase, in photosensitized
     erythrocytes: a novel thrombolytic system based on photodynamic
     activation.
AU   Flynn G; Hackett T J; McHale L; McHale A P
SO   JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY. B, BIOLOGY, (1994 Nov) 26 (2)
     193-6.

Thank you-

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Encapsulation of the thrombolytic enzyme, brinase, in photosensitized erythrocytes: a novel thrombolytic system based on photodynamic activation

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Abstract

In order to circumvent many of the problems associated with the systemic administration of agents used in thrombolytic therapy, it was decided to investigate the possibility of using erythrocytes as carriers and delivery vehicles for these agents. The enzyme brinase, a fibrinolytic enzyme produced by *Aspergillus oryzae*, was loaded into rabbit erythrocytes using electroporation. The loading index for this enzyme was found to be 60% and incorporation appeared to be relatively stable over a period of 4 h. In order to facilitate the predetermined release of the loaded component from the erythrocytes, they were photosensitized using haematoporphyrin derivative (HPD) and release was demonstrated within 5 min of photoactivation. Inclusion of the loaded, photosensitized system into clotting blood and subsequent exposure to light demonstrated almost complete lysis of the clot. We believe that this system exhibits potential for use in thrombolytic therapy.

Keywords: Brinase; Fibrinolytic; Thrombolytic; Erythrocytes; Photodynamic therapy; Electroporation

1. Introduction

Thrombolytic therapy, using agents such as urokinase, streptokinase and tissue plasminogen activator, has been successfully applied in the treatment of conditions such as arterial thromboembolism, cerebral vascular occlusive disease and acute myocardial infarction [1,2]. However, problems associated with the use of these agents include excessive bleeding with prolonged prophylactic use, inactivation of the thrombolytic activator by circulating protein inhibitors and possible elicitation of allergic reactions associated with multiple administration of non-compatible reagents such as streptokinase [3–5]. It has been proposed that brinase, a proteolytic enzyme produced by the fungus *Aspergillus oryzae*, and a thrombolytic agent, may find use in the clinic [6,7]. However, this enzyme has also been shown to be subject to inhibition by plasma protein inhibitors [8]. In addition, since brinase is of fungal origin, the possibility of the occurrence of adverse immunological reactions following systemic administration must be taken into account when considering it for use as a thrombolytic agent.

It would therefore appear that thrombolytic therapy in general would benefit from the development of some form of encapsulating carrier system which could be externally stimulated to release packaged thrombolytic activators at a predefined site. The use of erythrocytes seems to be an ideal choice as a carrier in this situation since they can be incorporated directly into thrombi during formation. In addition, the advantages associated with the use of erythrocytes as drug/active agent carriers are well documented [9].

We have previously demonstrated that photosensitized erythrocytes exhibit potential as carriers of photodynamic agents [10]. In this paper, we describe the loading of erythrocytes with the thrombolytic agent brinase. In addition, the loaded erythrocytes were photosensitized, and we demonstrate the photodependent release of brinase from the system. In in vitro studies, we demonstrate further that this system may be incorporated into clotting blood and photoactivation of the system brings about efficient thrombolysis. The potential advantages associated with the use of this system are discussed.

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2. Materials and methods

2.1. Brinase

Preparations of brinase were a gift from Professor P. Frisch, Royal College of Surgeons of Ireland, Dublin, Ireland. The highly purified preparation [6] consisted of a white lyophilized powder and was used as a 1 mg ml^{-1} stock solution which contained 10 units of activity as defined below. Activity was measured using an agar diffusion assay method based on a system reported previously [11]. Essentially, agar plates were prepared consisting of 1.5% (w/v) agar (Difco) in Hartman's dextrose (2.5% (w/v)) solution (HDS) containing 0.15% (w/v) fibrinogen and 0.5% (v/v) dog plasma. Wells (0.5 cm) were made in the agar. Samples to be tested for brinase activity were added to the plates and clearing zones were measured after predefined time periods. In this study, 1 unit of activity is defined as the amount of activity yielding a clear zone of 1 cm in diameter around each well after 1 h at 37 °C.

2.2. Loading of erythrocytes using electroporation

Rabbit erythrocytes (New Zealand White) were washed with HDS and resuspended to yield a 25% haematocrit. Erythrocytes (0.4 ml) were resuspended together with 0.4 ml aliquots of brinase (0.125 mg ml^{-1}). Electroporation was carried out using a Biorad gene pulser apparatus by placing 0.8 ml of the mixture into an electroporation cuvette (gap, 0.2 cm). Conditions of voltage and capacitance are given below. Following the delivery of the pulses, the cells were left at 3 °C for 12 h in order to facilitate resealing. Cells were then washed and the loading index was determined as the percentage of brinase associated with the erythrocytes, based on the amount of enzyme remaining following electroporation. The maximum amount of activity recovered following the hypoosmotic lysis of loaded erythrocytes was found to be 90% of the amount calculated to have been loaded. It is believed that this may have been due to the non-specific binding of enzyme to cell debris.

2.3. Photosensitization and photoactivation of the brinase-loaded system

Brinase-loaded cells were washed using HDS following electroporation and resealing. Cells were resuspended to yield a 15% haematocrit and 0.3 ml aliquots of the suspension were mixed together with haematoporphyrin derivative (HPD) (0.3 ml of a 250 μl ml^{-1} solution) which was synthesized as described previously [12]. Cells were incubated at 3 °C for 90 min after which they were washed twice in HDS. Photoactivation was accomplished by exposure to ra-

diation from a 10 mW HeNe laser (Hughes, CA, USA) placed 17 cm above the sample.

2.4. Assay of light-dependent lysis using the photosensitized, brinase-loaded erythrocytes

A 0.5 ml aliquot of [^{125}I]-labelled fibrinogen was incorporated into 1 ml of clotting whole blood together with a 0.3 ml aliquot of brinase-loaded, photosensitized erythrocytes. The resulting clot was then washed twice by centrifugation in HDS and subsequently suspended in 2 ml of HDS. It has been estimated that between 85% and 90% of the radioactivity was incorporated into the clot. Activation of the thrombolytic system was accomplished by exposure to irradiation for 5 min using the laser described above. The degree of clot lysis was determined by detection of the overall percentage radioactivity released into solution using a Packard 1500 scintillation counter.

3. Results and discussion

While it has been reported in the past that electroporation may be used to facilitate incorporation of substances into living cells [13], it was necessary to establish the optimal conditions for loading of brinase into erythrocytes. To this end, a variety of parameters were examined, including variations in voltage and capacitance. Some of the results obtained from these studies are shown in Fig. 1. While loading indices were relatively reproducible in all experiments, it was found that optimal loading indices in the region of 20% were observed following delivery of a single pulse at 1600 V using a capacitance setting of 1 μF (Fig. 1). However, subsequent experimentation demonstrated that delivery
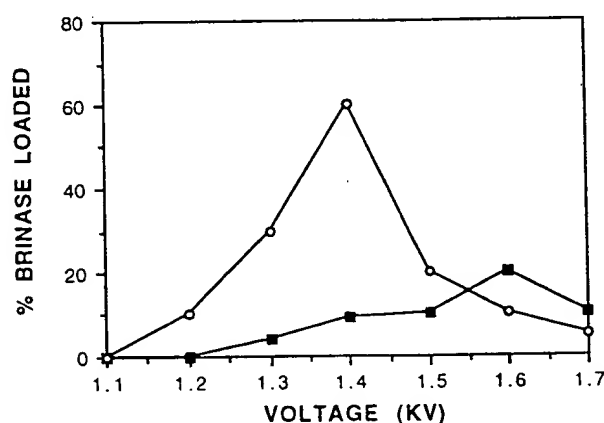

Fig. 1. Incorporation of brinase into erythrocytes using electroporation. Electroporation using single (■) and double (O) pulse delivery was carried out with a capacitance of 1 μF. The percentage loading indices were calculated by measurement of the activity remaining in solution following electroporation. (Standard deviation was less than 3% of the mean values.)

of a double pulse at 1400 V and 1 μF yielded a loading index of 60%. Therefore, in subsequent experimentation, the erythrocytes were loaded using the latter parameters. The loading indices reported here using electroporation compare very favourably with indices in the region of 40% previously reported using hypoosmotic dialysis methods [14]. Incubation of erythrocytes with brinase in the absence of an electroporation event, and subsequent estimation of the brinase content in solution following removal of the erythrocytes, demonstrated that at least 95% of the brinase could be recovered. This observation, taken in concert with that stated in Section 2.2 relating to the amount of enzyme recoverable following lysis using hypoosmotic shock, suggested that the majority of brinase associated with the erythrocytes following electroporation was incorporated into the cells and a relatively insignificant amount of the enzyme was actually bound to the cell surface. However, more extensive studies will have to be carried out in the future to establish conclusively whether or not this is the case.

In a previous report, it has been demonstrated that the photosensitization of erythrocyte ghosts and subsequent activation gives rise to disruption of these ghosts, and it was suggested that this might provide a suitable vehicle for photosensitizers for possible use in targeting the latter to predefined sites [10]. Therefore, since a mechanism was required for the release of the erythrocyte-entrapped brinase, it was decided to investigate the possibility of photosensitizing the loaded system and examining the light-dependent release of the enzyme from the cells. The results obtained from this series of experiments (Fig. 2) demonstrate that photodynamic activation provides a very convenient means of releasing the entrapped enzyme from the erythrocytes, with 90% of the entrapped enzyme re-

leased within 4 min following irradiation (Fig. 2). It should be noted that, in all cases, this was the maximum amount of activity that could be recovered from the erythrocytes, even if lysis of the cells was performed using hypoosmotic shock (see Section 2.2). The discrepancy observed between the calculated amount of enzyme loaded and that recovered following photoactivation may be due to the non-specific binding of the enzyme to the cellular debris which resulted following photolysis of the carrier vehicle. The results from this part of the study also demonstrate that the activation of the photosensitizer in the system does not contribute significantly to the inactivation of the enzyme activity released.

While it has been demonstrated that the thrombolytic enzyme can be incorporated into the erythrocytes with relatively high efficiency and that the system can be photosensitized in order to facilitate the light-dependent release of the loaded component, it is necessary to determine whether or not the system can be utilized to bring about clot lysis. In order to demonstrate the latter in vitro, it was decided to add the loaded, photosensitized system to clotting blood in order to facilitate intrathrombic incorporation. Subsequent exposure to light would then be expected to facilitate clot lysis. The results from these experiments are shown in Fig. 3. The study involved the measurement of the release of soluble [125I] which had been incorporated into the fibrin of the clot. It was found that up to 70% of the radioactivity, initially contained in the clot, was released into the supernatant within 10 min following irradiation, while only 8% was released in the absence of light. It was also found that exposure of clots containing the brinase-loaded erythrocytes to light, in
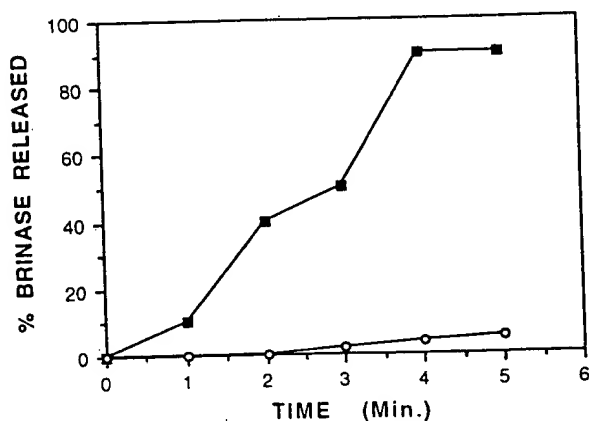

Fig. 2. Photoactivated release of brinase following irradiation of brinase-loaded, photosensitized erythrocytes. Aliquots (0.2 ml) of loaded and photosensitized cells were exposed to 4 min of irradiation (■) after which brinase release was measured. Control samples (O) consisted of the loaded, photosensitized system protected from light. (The standard deviation was less than 5% of the mean values.)
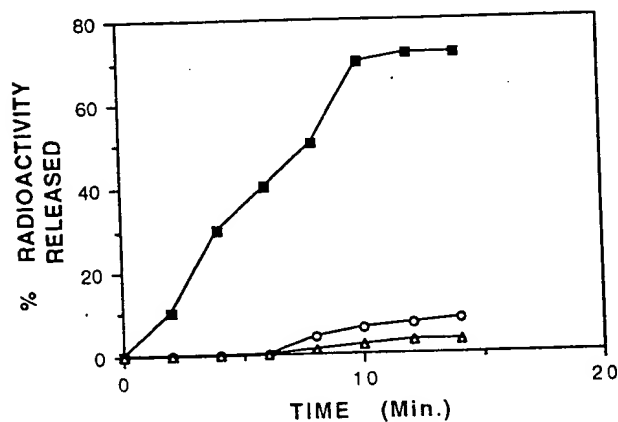

Fig. 3. Light-dependent release of radioactivity from clotted blood in which fibrin was labelled by the addition of [125I]-labelled fibrinogen. Radioactivity released from clots containing the loaded, photosensitized system following exposure to (■) and protection from (O) light was measured as described in Section 2. The release of radioactivity from clots containing the loaded erythrocytes in the absence of photosensitizer but exposed to light was also measured (△). (The standard deviation was less than 4% of the mean values).

the absence of photosensitizer, failed to bring about the significant release of radioactivity from clotted blood. In all experiments carried out to date, visual examination of the clots containing loaded, photosensitized erythrocytes exposed to light indicated almost complete lysis. It should be noted here that incubation of the photosensitized, loaded erythrocytes in the clot for periods of up to 3 h, in the absence of light, failed to release greater than 10% of the incorporated radioactivity. This result suggests that the erythrocyte-entrapped brinase is relatively stable and inactive in the absence of light. It also suggests that if brinase is associated with the cell surface in the system, this is of relatively little consequence.

Although the use of erythrocytes as drug/active agent carriers has previously been reported [9,14–16], the release of the active agent from the system has depended on passive release of the encapsulated material from the cells. The choice of erythrocytes as a carrier of the thrombolytic enzyme brinase in this study was of particular significance, as they would be expected to be trapped within forming thrombi, and many of the advantages associated with intrathrombic thrombolytic therapy would apply [17]. An extension of previous work in our laboratory using erythrocytes as carriers of photosensitizers [10] suggested that photodynamic activation would provide a suitable system for facilitating rapid, predefined release from the system. The results presented here demonstrate this to be the case. In considering this system for use in vivo, it would be envisaged that it may be an alternative to conventional prophylactic use of thrombolytics. In a formed or forming thrombus, the system could be activated by delivery of light to deep sites using fibreoptic devices.

Although the findings reported here may be considered to be relatively preliminary, we believe that in overall terms, with respect to thrombolytic therapy, advantages associated with the system include prevention of inhibition of the thrombolytic agent by circulating inhibitors [3], protection against adverse immunological responses associated with prolonged exposure to foreign thrombolytic agents [3], decreasing excessive bleeding [4] and increasing half-life of the thrombolytic agent in circulation [5]. From the point of view of the use of this system for general applications in drug/active agent delivery/targeting, we believe that the combined use of electroporative loading of erythrocytes and subsequent photosensitization in order to facilitate predefined release from the system represents a novel approach with extraordinary potential.

Acknowledgements

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Electric field-enhanced activation of hematoporphyrin derivative: effects on a human tumour cell line

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Abstract

In a recent report we described the effects of combined electroactivation and photoactivation of hematoporphyrin derivative (HPD) on human erythrocytes and established that activation-induced cell lysis was more pronounced when both modes of activation were sequentially applied to the system. Here we demonstrate that electric field-induced activation of HPD-treated HeLa cells results in cell death. This effect is shown to be dependant on both electric field strength and on HPD concentration. In addition, we demonstrate that exposure of HPD-treated cells to short and intense electric pulses prior to photoactivation, results in increased cell mortality. The results confirm our earlier suggestion that HPD may be activated in the presence of an applied electric field. The results further suggest that activation of photosensitizers using combined exposure to electric fields and light may play an important role in increasing the efficiency of photodynamic therapy (PDT) in the treatment of cancer. © 1997 Elsevier Science Ireland Ltd.

Keywords: Hematoporphyrin derivative; Electric; Fields; Cancer; Photodynamic; Therapy; HeLa

1. Introduction

Photodynamic therapy (PDT) in which two relatively harmless agents, photosensitizer and light, are combined to bring about a cytotoxic effect has been described as a promising new modality for the treatment of cancer' [1]. To date regulatory agency clearance has been obtained for the clinical use of Photofrin® in a number of countries including Canada, the Netherlands, Japan and the USA [1]. Clinical areas for which approval has been obtained include superficial bladder cancer, lung cancer, skin cancer and cancers of the upper aerodigestive tract [1]. Although a variety of alternative photosensitizers exist including benzoporphyrin derivative monoacid ring A, mono-1-aspartyl chlorin e6 and etiopurpurin, their use in the clinic awaits demonstration that they are at least as effective as Photofrin®. Although hematoporphyrin derivative (HPD)-based therapy is the most characterized form of PDT to date, its clinical acceptance appears to be hindered by a number of side effects associated with conventional therapy. These include problems associated with clearance of the photosensitizer by the liver [2], development of skin

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photosensitization for prolonged periods of time [3] and in some cases, incomplete tumour eradication as a result of reduced light transmission through living tissues [1]. It is currently believed that the latter problem may be overcome by using some of the above listed alternative photosensitizer preparations since they absorb light in the 650–690 nm region of the spectrum. It is, however, possible that this problem could also be alleviated by increasing the activation efficiency of the clinically approved Photofrin® at the appropriate target site. Achieving this objective would also facilitate increasing the efficiency of those novel second generation photosensitizers currently under investigation.

In recent years is has been demonstrated that it is possible to apply short and intense electric pulses in vivo to achieve transient permeabilization of cells without adversely affecting the subject [4]. This phenomenon has been exploited in developing a relatively novel means of cancer treatment know as electrochemotherapy [5]. Many of the earlier studies were concerned with achieving transient permeabilization of tumour cells to agents such as bleomycin, particularly in bleomycin-resistant disease where access of the active agent to the target was limited by the lack of a cellular uptake mechanism [6]. More recently clinical studies involving the use of electrochemotherapy together with bleomycin in the treatment of cancers such as malignant melanoma, basal cell carcinoma and metastatic adenocarcinoma, concluded that the treatment modality was quite effective in the majority of sites treated [7]. The studies also concluded that patients tolerated the treatment well with no residual side effects from the electric pulses.

Many of the studies in our laboratories have been directed towards the development of specific targeting/carrier systems for photosensitizing agents [8,9]. In more recent studies it has been shown that erythrocytes may be used as carriers of active agents such as chemotherapeutics [10] and enzymes [11] and light-dependent release of those agents may be achieved by photosensitizing the cell membrane and subsequent exposure to laser radiation. In those studies it was found that very efficient loading of the erythrocytes may be achieved by electro-permeabilizing the cells. In a more recent study it was found that if the cells are treated with the photosensitizer HPD and subse-

quently exposed to electric pulses, a rapid lysis of the cells occurred [12]. This effect was dependent on electric field strength and on the concentration of photosensitizer. Using confocal laser scanning microscopy to study this effect it was found that the lytic event was similar to that observed when photosensitized erythrocytes were exposed to light alone [13]. In addition, it was found that by exposing photosensitized erythrocytes to a combination of light and electric pulses, the efficiency of lysis increased dramatically. The results from that study suggested that the photosensitizer, HPD, was being activated by exposure to the electric pulses and this further suggested that combined exposure of any photosensitized target to light and electric pulses would enhance PDT. In the study presented here it was decided to determine whether or not electric field-induced activation of the HPD-treated human tumour cells would result in a cytotoxic event. It was also decided to examine the effects of exposing the photosensitized tumour cell line to a combination of light and electric pulses. Based on the results obtained here we suggest the possible advantages associated with the use of combined light- and electric field-induced activation of photosensitizers during PDT.

2. Materials and methods

2.1. Cell line and growth conditions

The HeLa cell line was obtained from the European Collection of Animal Cell Cultures (ECACC no. 85060701) and was maintained on minimal essential medium (Gibco) supplemented with Earle's balanced salt solution (Gibco), 1% (v/v) non-essential amino acids (Gibco) and 10% (v/v) foetal bovine serum (Gibco). Cells were grown in a 5% CO_2 humidified atmosphere at 37°C. Cells were harvested by centrifugation following treatment with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in phosphate buffered saline (PBS) for 5 min at 30°C. Prior to plating or manipulation, cells were counted using a haemocytometer and cell viability was assessed using the trypan blue dye exclusion assay [14].

2.2. Photosensitization of cell populations

HPD was prepared as described previously [15].

Cells were photosensitized by incubation in medium containing the required concentration of HPD for 1 h. Cells were then washed in medium by centrifugation at 400 × *g* for 5 min and subsequently resuspended in the appropriate medium.

2.3. Irradiation parameters

Cells to be treated were aliquoted into the wells of a 96-well plate and irradiated using a 10 mW output HeNe laser, placed 17 cm above the samples to be treated. The power density was determined to be 43 mW/cm². Following treatment, cell viability was assessed using the MTT-based cell viability assay described previously [14].

2.4. Delivery of electric pulses

Cells to be treated with electric pulses were suspended in 0.6–0.8 ml of medium, dispensed into electroporation cuvettes (electrode gap 0.4 cm) and electric pulses were delivered using a BioRad Gene Pulser (UK) apparatus. In all cases samples were exposed to single pulses and the time constant varied between 3 and 8 ms. Following treatment, cells were aliquoted into the wells of a 96-well plate and incubated at 37°C for the required period of time. Following treatment cell viability was assessed using the MTT cell viability assay as described previously [14].

3. Results

3.1. Effect of increasing the electric field strength on viability of photosensitized HeLa cells

Since it had been demonstrated previously that exposure of photosensitized erythrocytes to electric pulses with increasing field strength resulted in increased cell lysis [12] it was decided to determine whether or not similar conditions of exposure would adversely affect the viability of photosensitized HeLa cells. To this end HeLa cells were photosensitized with HPD (0.6 mg/ml) and subjected to single electric pulses with field strengths ranging between 75 V/cm and 1450 V/cm at 25 μF. Control cell populations consisted of non-photosensitized cells exposed to electric pulses. Following treatment cells were plated

for 24 h and the cell viability was determined using the MTT assay [13]. The results are shown in Fig. 1 and they clearly demonstrate increased cell mortality in the presence of photosensitizer. Cell viability decreased to zero at 725 V/cm at 25 μF whereas cells treated with electric pulses in the absence of photosensitizer still exhibited 40% viability. Although significant effects were observed when the non-photosensitized cells were exposed to pulses of increasing electric field strength, the cell populations did eventually recover to their original pre-treated cell density (results not shown). Cells treated with the chosen concentration of photosensitizer and electric field strengths above 725 V/cm failed to recover. It should be noted that the viability of photosensitized cells in the absence of an applied electric field remained unchanged at the concentration of photosensitizer used.
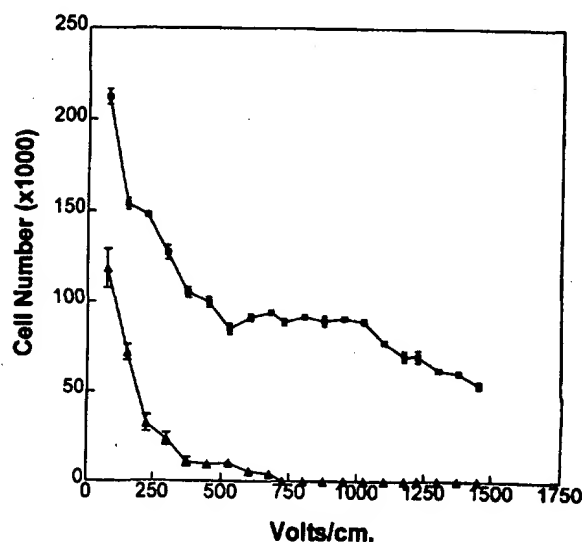

Fig. 1. The effect of increasing electric field strength on the viability of HPD-treated HeLa cells. HeLa cells (2.5 × 10⁵) were photosensitized with HPD (0.6 mg/ml) (△) and suspended in 0.6 ml of medium. Cells were placed in an electroporation cuvette and exposed to electric pulses (capacitance 25 μF) with the indicated field strengths. Cells were then recovered by centrifugation and subsequently plated. Cell viability was assessed using the MTT assay described in Section 2. The control samples (■) consisted of non-photosensitized cells (2.5 × 10⁵) exposed to electric pulses at the indicated field strengths. The data reflect the mean values ± SE from five experiments.

3.2. The effect of HPD concentration on electric field-induced mortality of HeLa cells

In order to determine whether or not the effect demonstrated above was dose-dependent with respect to HPD concentration, cells were photosensitized in various concentrations ranging from 0 to 3 mg HPD/ml. Cells were then exposed to electric pulses with an electric field strength of 300 V/cm at 250 μF. Cells were plated and viability was determined 24 h after treatment using the MTT assay. The results obtained are shown in Fig. 2 and they demonstrate a rapid decrease in cell viability with increasing concentration of photosensitizer. Increasing the HPD concentration from 0 to 1.5 mg/ml had almost no effect on cell viability in the absence of an applied electric field. Above 1.5 mg/ml, however, a slight decrease in cell viability was observed in those control samples and this was attributed to dark toxicity of the HPD preparations. It should be noted that when populations of cells were treated with 300 V/cm at 250 μF in the absence of photosensitizer, a 10% decrease in cell viability was observed and this did not significantly affect the result shown in Fig. 2.
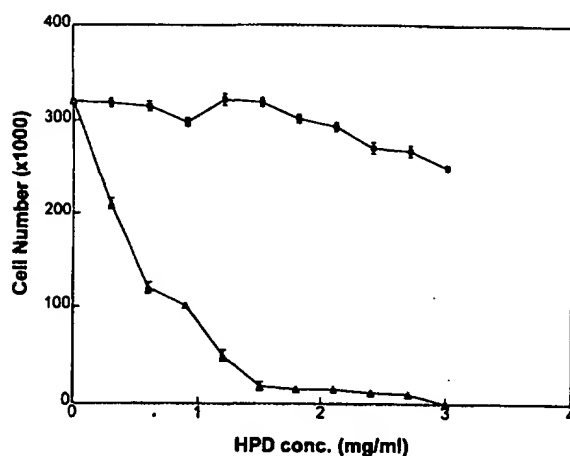

Fig. 2. The effect of HPD concentration on the viability of HeLa cells exposed (Δ) to electric pulses (field strength 300 V/cm at 250 μF). Cells were photosensitized with HPD and exposed to electric pulses as described for . Control samples (■) consisted of HPD-treated cells which were not exposed to an electric field. The cell viability was determined using the MTT assay as described. The data reflect the mean values ± SE from five experiments.
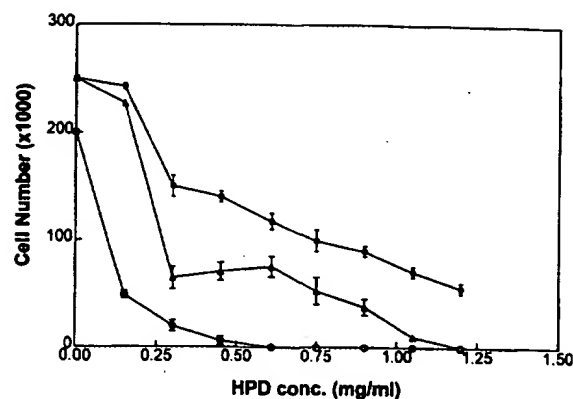

Fig. 3. The effects of combined light and electric field exposure on the viability of photosensitized HeLa cells. Cells were photosensitized using the concentrations of HPD indicated. Preparations of HPD-treated cells were exposed to 6 min irradiation (■) using a HeNe laser. Photosensitized cells were also exposed to electric pulses (field strength 300 V/cm; 250 μF) (Δ). In addition, samples were first exposed to electric pulses and exposed to 6 min irradiation from the HeNe laser (●) within 10 min of delivering the electric pulse. In all cases cell viability was determined using the MTT assay described previously [15]. Data points reflect the mean values ± SE from six experiments.

3.3. The combined effects of electric pulses and light on viability of HPD-treated HeLa cells

We have already demonstrated that stimulation of HPD by combined exposure to electric pulses and light results in increased lysis of photosensitized erythrocytes [12]. In order to determine whether or not combined treatment of the photosensitized HeLa cells would lead to a more pronounced decrease in cell viability, it was decided to expose photosensitized cells to the electric pulses and subsequently to expose those cells to light from a low-powered HeNe laser. These experiments were carried out at different concentrations of photosensitizer and cell viability was determine 24 h after the relevant treatment using the MTT assay. Cells were exposed to electric pulses with a field strength of 300 V/cm at 250 μF and as expected the cell viability decreased with increasing concentration of HPD (Fig. 3). Photosensitized cells were also irradiated for 6 min using a 10 mW HeNe laser as described above and again, cell viability decreased with increasing concentration of HPD (Fig. 3). When photosensitized cells were exposed to electric pulses and subsequently to light a dramatic decrease in cell

viability was observed, particularly at lower concentrations of photosensitizer (Fig. 3). It should be noted that under the conditions of the experiments, cells treated with combined electric pulses and light at concentrations of HPD above 0.6 mg/ml failed to recover, whereas cell populations treated with either electric pulses or light alone at this concentration of HPD recovered to their original pre-treatment cell densities. It would appear that at lower HPD concentrations an effect greater than the combined effects of both treatments was observed. This suggested some form of synergistic action although establishing synergy awaits further, more complex data analysis. In any event it would appear from the results presented here that combined exposure of photosensitized HeLa cell populations to electric pulses and light results in an overall decrease in cell viability, and potential offered by this observation in cancer therapy is discussed further below.

4. Discussion

Exposure of cells to short and intense electric pulses usually brings about a membrane permeabilization event which appears to be reversible in most cases [4]. This phenomenon has been exploited in molecular biology where exposure of cells to electric pulses facilitates cell transformation [16]. It has also been used in studying the effects of permeability on modified cellular metabolism [17]. In our laboratories it has been exploited to achieve efficient and reproducible loading of human erythrocytes with active agents such as cancer chemotherapeutics and enzymes [10,11]. With respect to exploitation of this technology in vivo, it has been demonstrated that delivery of electric pulses to sites in internal organs facilitates in vivo gene transfer [18]. In addition, it has been demonstrated that applying electric fields across a target tissue results in in vivo electropermeabilization and this has contributed to the development of what has become known as electrochemotherapy. Electropermeabilization of bleomycin-resistant tumours results in increasing susceptibility of the disease to that drug by facilitating its entry into the cell [6]. In a number of clinical trials significant responses to electrochemotherapy have been reported without any reported adverse effects associated with exposure to the electric pulses [7,19].

Recently we reported that exposure of photosensitized human erythrocytes to electric pulses resulted in an apparent increased susceptibility of those cells to photodynamic activation [12]. In that report it was shown that combined exposure of photosensitized cells to electric pulses and light resulted in a degree of cell lysis which was greater than that induced by either stimulus separately. One hypothesis suggested that the cell membrane became destabilized by exposure to the electric pulses rendering those erythrocytes more susceptible to light-induced lysis. An alternative hypothesis suggested possible molecular energy redistribution induced by the applied electric field. Such a redistribution and subsequent relaxation to the ground state could allow for the formation of 'singlet oxygen' in much the same manner proposed for photodynamic activation [12]. Although the exact reason for enhanced activation was, and still is, unknown the results did demonstrate an increased degree of cell lysis.

In order to determine whether or not our observations with photosensitized erythrocytes could be translated to human tumour cells it was initially decided to study the effects of exposing HPD-treated HeLa cells to electric pulses in the absence of light. The results shown in Fig. 1 demonstrated that exposure of HPD-treated cells to electric pulses resulted in a dramatic decrease in cell viability. It was also shown that this effect was dose-responsive with respect to the electric field strength. Although control populations of cells which were not treated with HPD also exhibited a decrease in cell viability it was found that cells did eventually recover to their original cell density when treated with field strengths above 750 V/cm. HPD-treated cells failed to exhibit any degree of viability when field strengths above 750 V/cm were been applied. These results suggested that some form of activation event was occurring during the application of the electric pulses and confirmed our earlier observations with photosensitized erythrocyte populations [12].

As with our studies using electric field-induced activation of HPD-treated erythrocytes it was decided to determine whether or not the observed effects with the HeLa cells were dose-responsive with respect to HPD concentration. The results shown in Fig. 2 demonstrate a clear relationship between HPD concentration and a reduction in cell viability, particu-

larly at lower concentrations of HPD. Again, the results suggest some form of 'dark' activation event by exposing HPD to electric pulses.

Since our previous studies demonstrated enhanced lysis of photosensitized erythrocytes exposed to both stimuli, it was decided to determine whether or not exposure of the HPD-treated HeLa cells to combined electric pulses and light would result in an overall decrease in cell viability. The results shown in Fig. 3 demonstrate that separate delivery of light or electric pulses alone to the system resulted in a decrease in cell viability. It was interesting to note that electro-activation had the greater effect on the viability of the target cells. This general observation is in close agreement with our work relating to the effect of electric fields on photosensitized erythrocytes [12]. In that case it was found that lysis of photosensitized erythrocytes following stimulation by exposure to electric fields was greater than that observed following exposure to light.

When the HPD-treated HeLa cells were exposed to both stimuli a dramatic increase in the effect was observed, particularly at lower concentrations of HPD. At concentrations of HPD above 0.6 mg/ml, cell viability remained at 0 when both stimuli were applied, whereas cell densities eventually recovered in samples treated with each stimulus separately. Although the exact mechanism of electric field-induced activation of HPD is as yet unknown, preliminary work in our laboratories suggests the formation of 'singlet oxygen' during exposure of the HPD to the electric fields. It has also been shown that this is not derived from the electrodes during delivery of pulses. More detailed confirmation of these observations is currently under way in our laboratories. The results presented here again confirm our earlier observations with photosensitized erythrocytes and they suggest a possible clinical role for combined electric field- and light-induced stimulation of HPD in photo-dynamic therapy.

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